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Inhibition of superoxide anion release from circulating neutrophils by L-arginine in man

C.J. Wiedermann, B. Sitte, U. Zilian, N. Reinisch, H. Beimpold, G. Finkenstedt, H. Braunsteiner
Universitätsklinik für Innere Medizin, Medizinische Fakultät, Universität Innsbruck

Summary. In animal studies of myocardial ischemia/reperfusion L-arginine reduces necrotic injury by preservation of endothelial function and attenuation of neutrophil accumulation in ischemic cardiac tissue. Because release of oxygen radical species by circulating neutrophils is important in endothelial function and ischemia-reperfusion injury, this study investigated the effect of intravenous administration of L-arginine on the in vitro release of superoxide anion of neutrophils in healthy young adults. Neutrophils were obtained at various time points before, during, and after infusion of L-arginine (17 mg kg^{-1} min^{-1} for 30 min) and analyzed for superoxide dismutase inhibitable reduction of ferricytochrome *c*. The spontaneously occurring respiratory burst of polymorphonuclear leukocytes at basal conditions was compared with that after triggering by 1 μmol/l formylpeptide or 50 ng/ml phorbolester. Infusion of L-arginine inhibited both basal ($P < 0.01$) and formylpeptide-triggered ($P < 0.05$) release of superoxide anion did, but not affect release stimulated by phorbol 12-myristate 13-acetate. Pretreatment of neutrophils with 1 mmol/l L-arginine in vitro also significantly reduced formylpeptide-triggered (1 μmol/l) superoxide anion release, suggesting that the affects observed after in vivo pretreatment may be due to direct action of L-arginine on neutrophils. These findings demonstrate the ability of L-arginine to reduce release of oxygen radical species by circulating neutrophils in man.

Key words: Nitric oxide – Respiratory burst – Ischemia – Reperfusion

L-Arginine has been shown to be a precursor of nitric oxide (NO). NO accounts for the biological properties of endothelium-derived relaxing factor [11]. NO is widely believed to play an important role in vascular homeostasis including the functions of leukocytes and platelets [11]. Recent results demonstrate the ability of L-arginine to reduce necrotic injury in a cat model of myocardial ischemia-reperfusion, and this reduction in infarct size was associated with the preservation of endothelium function and attenuation of polymorphonuclear leukocyte (PMNL) accumulation in ischemic cardiac tissue [16]. In intact animals, studies using inhibitors of NO synthetases have shown that endogenous NO derived from L-arginine reduces activation of PMNL and platelets [2, 5, 10]. Thus, increased production by PMNL of NO after administration of L-arginine may have contributed to the attenuated accumulation in tissues of ischemia-reperfusion injury. It is as yet unknown whether in man L-arginine as a regulator of neutrophil and platelet functions has any role to play. We therefore investigated the in vivo effects of exogenous L-arginine on spontaneous and stimulated superoxide anion release from PMNL in man.

Abbreviations: HBSS = Hank's balanced salt solution; FMLP = formyl-Met-Leu-Phe; NO = nitric oxide; PMA = phorbol 12-myristate 13-acetate; PMNL = polymorphonuclear leukocytes; SOD = superoxide dismutase

Materials and methods

Materials

Materials utilized in this study included phorbol 12-myristate 13-acetate (PMA) and formyl-Met-Leu-Phe (FMLP; Sigma, St. Louis, MO). FMLP was stored at −20°C in dimethylsulfoxide at a concentration of 10 mmol/l and was diluted into assay medium prior to use. PMA was reconstituted to 1 mg/ml in ethanol and also stored at −20°C. Ferricytochrome *c* and superoxide dismutase (SOD) were from Sigma and Hanks' balanced salt solution (HBSS) from Gibco (Vienna, Austria).

Study subjects

Eight healthy subjects (seven men, median age 29 years, range 22–38; one woman, age 23 years) participated on a voluntary basis after the procedure

of the study had been fully explained and informed consent obtained. None of the male subjects were on medication; the female subject was on oral contraception. Physical activity or diet was not monitored. The eight subjects were normocholesterinemic and had normal test results on routine laboratory measures and physical examination. At 10.00 a.m., after a 10-min bedrest, infusion into an antecubital vein of L-arginine-HCl (Braun, Maria Enzersdorf, Austria) at 500 mg/kg body weight in 250 ml physiological saline at a rate of 17 mg kg^{-1} min^{-1} was started, which lasted for a total of 30 min. Peripheral venous blood samples were taken at time 0, just before the infusion was started, and then at 15, 30, 45, 60, and 90 min after the infusion was started. After collection of samples, which were kept at room temperature, had been completed, they were immediately processed for further analysis, including routine laboratory tests for blood glucose, electrolytes, blood gas and hormone levels for prolactin, growth hormone, and insulin-like growth factor I. Hemodynamic parameters were monitored automatically every 10 min with the cuff at the upper left arm. Plasma levels of growth hormone and prolactin were measured using commercially available radioimmunoassay kits (HGHK-2, Sorin Biomedica, Saluggia, Italy; IMX-System Prolactin, Abbott Laboratories, Abbott Park, IL). Plasma samples were stored at −80°C until collection from all subjects had been completed, and processed as recommended by the manufacturers.

As described previously [13], infusion of L-arginine caused a rapid onset of hypotension. The reduction in mean arterial pressure was transient with statistically significant changes seen at 30 min of observation (before L-arginine 94.3 ± 3.01 mmHg, at 30 min, 85.9 ± 2.86 mmHg; analysis of variance repeated measures, $P < 0.005$, $n = 7$). Since at the same time the heart rate of the subjects did not significantly fall ($n = 7$, $P > 0.1$), it is likely that L-arginine induced hypotension was caused by vasodilatation. No changes were observed in blood gas, glucose, or sodium levels. Potassium levels increased slightly from 3.7 ± 0.12 mmol/l before L-arginine to 4.6 ± 0.30 mmol/l at 90 min of observation. At 90 min, the change of potassium levels was statistically significant ($n = 4$; Mann-Whitney U test, $P < .05$). L-arginine caused a charcteristic rise in plasma levels of growth hormone and prolactin, which peaked at 60 min and 45 min, respectively (growth hormone levels in plasma before L-arginine administration 2.9 ± 2.06 ng/ml, at 60 min, 9.8 ± 2.87 ng/ml, $n = 8$, $P < 0.05$; prolactin before L-arginine administration 6.1 ± 0.88 ng/ml, at 45 min 14.2 ± 2.36 ng/ml; $n = 8$, $P < 0.05$, two-tailed t test for paired samples).

Neutrophil preparation

From the peripheral blood of subjects under study (anticoagulated with ethylenediaminetetraacetate), PMNL were obtained after discontinuous density gradient centrifugation of whole blood on Percoll as described [19], followed by lysis of contaminating erythrocytes using hypotonic sodium chloride solution. The cell preparations (> 95% PMNL by morphology in Giemsa stains, > 99% viable by trypan dye exclusion) after centrifugation were resuspended in HBSS. Storage of whole blood at room temperature for up to 6 h did not affect functional responses of PMNL in the assay as far as baseline and triggered respiratory burst activity are concerned (data not shown).

Superoxide anion release

The production of O_2^- was assayed by the reduction in ferricytochrome *c*, the specificity of reduction being controlled by its inhibition by SOD. Immediately after preparation of PMNL, 2×10^6 PMNL/ml (100 µl/well) was immersed in a 160 µM solution of ferricytochrome *c* in phenol red-free HBSS containing 1 µM FMLP, 50 ng/ml PMA, or vehicles. To one vertical row ferricytochrome *c* and 300 U/ml SOD were added. This row served as a blank. The plates were covered with lids and placed in a 37°C humidified incubator with 95% air, 5% CO_2 atmosphere. At 10-min intervals of incubation the plates were transferred to the enzyme-linked immunosorbent assay reader (Labsystems, Helsinki, Finland), and absorbances were read at 550 nm [19].

In initial experiments, ferricytochrome *c* reduction by PMNL before, during, and after a 30 min infusion of normal saline was tested, and no significant alteration was observed (data not shown). Therefore, statistical analysis of the effects of L-arginine infusion on PMNL superoxide anion release was performed by comparison with pretest values.

Neutrophil treatment by L-arginine in vitro

Neutrophils prepared from forearm venous blood from untreated donors were used for investigating the effect of L-arginine in vitro. For coincubation studies 1 mmol/l L-arginine was present in incubation medium during the ferricytochrome *c* reduc-

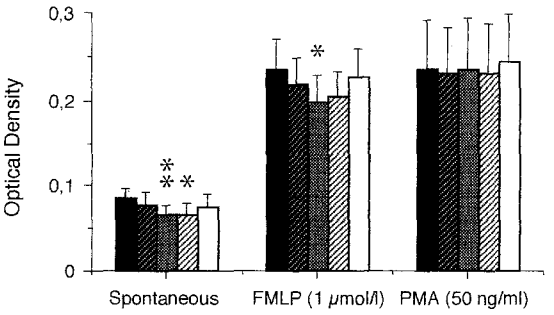

Fig. 1. Effect on basal and stimulated superoxide anion release from neurophils of intravenous infusion of 17 mg kg^{-1} min^{-1} L-arginine for 30 min in man. Superoxide anion release was measured by SOD-inhibitable reduction of ferricytochrome *c*. Each point is the mean ± SE, $n = 8$. * $P < 0.05$ (two-tailed Student's *t* test for paired samples) compared to corresponding time control. ■ 0 min; ▨ 30 min; ▨ 45 min; ▨ 60 min; □ 90 min

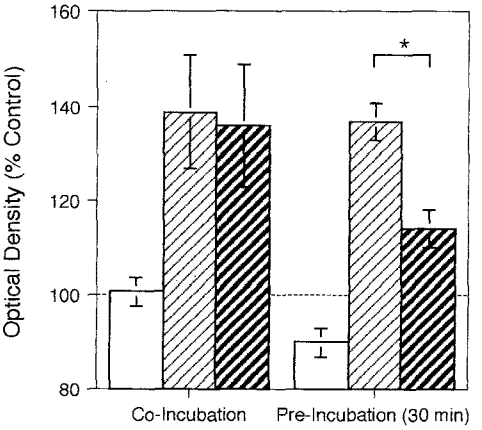

Fig. 2. Effects of L-agrinine on basal and FMLP (1 μmol/l) stimulated superoxide anion release from neutrophils in vitro. Superoxide anion release was measured by SOD-inhibitable reduction of ferricytochrome *c* at 10 min of incubation during the simultaneous presence of 1 mmol/l L-arginine in the incubation medium (*co-incubation*) or after 30 min of pretreatment of neutrophils with 1 mmol/l of L-arginine (*pre-incubation*). Each point is the mean ± SE, $n = 4$ and $n = 7$ in co-incubation and pre-incubation studies, respectively; * $P < 0.05$ (*U* test). □ L-Arginine (1 mmol/l); ▨ FMLP (1 μmol/l); ▨ L-Arginine plus FMLP

tion assay as described above. Preincubation experiments were performed using 2×10^6 PMNL/ml in HBSS containing 1 mmol/l L-arginine. Cells were incubated at 37°C for 30 min, then centrifuged and resuspended in HBSS for assaying ferricytochrome *c* reduction.

Results

The interference of L-arginine with respiratory burst activity of PMNL was assessed by measuring the reduction of ferricytochrome *c* at various time points before, during, and after the infusion of L-arginine (Fig. 1). L-Arginine caused a transient decrease in baseline respiratory burst activity of PMNL, which paralleled in time the secretagogue effects of L-arginine on pituitary growth hormone and prolactin (see above). Respiratory burst was also triggered by exposure of PMNL to 1 μmol/l FMLP or 50 ng/ml PMA. It was found that L-arginine administration significantly suppressed respiratory burst activity of PMNL when FMLP was used as a triggering agent; no effect of L-arginine was seen with PMA as stimulus. The effects of L-arginine on basal and FMLP-triggered respiratory burst were maximal at 45 min of observation.

To study direct effects of L-arginine we tested the influence of L-arginine in vitro on reduction of ferricytochrome *c* by PMNL in coincubation and pretreatment studies (Fig. 2). A combination of 1 mmol/l L-arginine with 2×10^6 PMNL/ml for up to 30 min did not affect basal or formylpeptide-triggered (1 μmol/l) reduction of ferricytochrome *c*. After pretreatment of 2×10^6 PMNL/m l in HBSS with 1 mmol/l L-arginine at 37°C for 30 min, however, a significant abrogation of formylpeptide-triggered (1 μmol/l) reduction in ferricytochrome *c* was observed.

Discussion

NO formed from L-arginine has previously been demonstrated to have an effect on phagocyte cytotoxicity. It has been shown that decrease in L-arginine concentrations at the site of wounds or at other sites of inflammation impairs macrophage cytotoxicity but enhances other macrophage activation-associated functions [1]. Findings have suggested interactions between NO formation and mechanisms of macrophage activation (for review see Moncada et al. [11]). In PMNL the formation from L-arginine of NO by the NO synthetase has been unequivocally demonstrated [9]. Yet, the biological significance of the production of NO by PMNL remains to be elucidated. There is no evidence that PMNL-derived NO plays a role in the cytotoxic activity of these cells [11].

In this study we have found that intravenous administration of L-arginine inhibits basal and FMLP-stimulated superoxide anion release, as measured after isolation of PMNL from forearm venous blood. No effect of L-arginine was seen when PMA was used as triggering agent for PMNL respiratory burst. Activation of the respiratory burst involves several steps. The protein kinase C activation and elevation of free Ca^{2+} are induced

by G protein dependent activation of FMLP receptors and contribute to the activation of the "respiratory burst enzyme" NADPH oxidase [6]. In contrast to FMLP, PMA activates protein kinase C directly [14], which can explain why the PMA-induced respiratory burst typically is not affected by PMNL-activating compounds that act via the receptor–G protein complex. Infusion of L-arginine inhibited basal and FMLP-induced but not PMA-induced respiratory burst activity of PMNL, suggesting modification of the receptor–G membrane protein complex by L-arginine induced events in PMNL.

The inhibition of FMLP-stimulated superoxide anion release by L-arginine in vivo was also seen when a corresponding concentration of L-arginine (1 mmol/l) was added to PMNL in pretreatment studies in vitro. As compared to the minor effect of L-arginine after in vivo administration, pretreatment of PMNL in vitro resulted in a more potent inhibition of 1 μmol/l FMLP-triggered superoxide anion release. The data suggest that L-arginine directly affects PMNL superoxide anion release.

The time course of the effects on basal and FMLP-triggered PMNL activity parallels the endocrine changes seen after infusion of L-arginine. Effects of L-arginine on hemodynamics occurred 15–30 min earlier than on endocrine changes and superoxide anion secretion by PMNL. It has recently been reported that growth hormone and prolactin can prime PMNL for enhanced respiratory burst activity in response to various triggering agents [4, 17, 18]. As growth hormone and prolactin are released by infusion of L-arginine, theoretically one would expect augmentation of PMNL activity to occur. Contrary to expectations, however, inhibition of PMNL function was observed, suggesting that release of growth hormone is not the explanation for the effects of L-arginine on PMNL function.

As with macrophages, PMNL metabolize L-arginine via an oxidative pathway that generates NO, nitrate, and nitrite, the synthesis of NO being enhanced by stimulation of PMNL with the chemoattractants FMLP and leukotriene B_4 [8]. Superoxide anions, which are induced concomitantly in PMNL, however, interact with NO such that the levels of biologically active NO decrease as superoxide anion production increases [8]. In vitro L-arginine enhances the release of NO observed in the presence of FMLP [8]. Treatment of PMNL with SOD enhances the biological action of NO released by FMLP, as does L-arginine [9]. NO has been shown to inhibit PMNL aggregation in vitro [7] and the NO-producing agents molsidomine and 3, morpholino-sydnonimine to inhibit lysosomal enzyme release from PMNL [15]. Recently, Clancy and co-workers [3] have demonstrated that NO inhibits superoxide anion production via direct action on the membrane components of NADPH oxidase only when added to PMNL 20 min before FMLP, presumably through formation of stable intermediate of NO. If exogenous L-arginine augmented NO in our study subjects, our data would be consistent with an inhibitory effect on respiratory burst of PMNL by the L-arginine: NO pathway in vivo. In this study circulating PMNL were treated with L-arginine in vivo, then isolated from peripheral blood by density gradient centrifugation and several washing steps. Thereafter, basal and triggered reduction of ferricytochrome *c* was assayed. This experimental procedures may have allowed some PMNL to recover from the effects of L-arginine treatment and thus may explain the relatively minor though significant effect of L-arginine given in absolute terms (at 45 min, 77.5 $\pm$ 5.69% of pretest values for basal superoxide anion release and 86.3 $\pm$ 6.17% of pretest values for FMLP-triggered superoxide anion release). In contrast, a powerful action of L-arginine was seen in corresponding studies in vitro.

The results of this study demonstrate the ability of L-arginine to inhibit PMNL superoxide anion release in man.

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Univ.-Doz. Dr. C.J. Wiedermann
Medizinische Klinik
Anichstrasse 35
A-6020 Innsbruck
Austria